

# Peptide-mediated cellular delivery

## John J Schwartz & Shuguang Zhang

### Address

Center for Biomedical Engineering 56-341  
Massachusetts Institute of Technology  
77 Massachusetts Avenue  
Cambridge  
MA 02139-4307  
USA  
Email: jschwartz@MIT.EDU  
shuguang@MIT.EDU

Current Opinion in Molecular Therapeutics (2000) 2(2):162-167  
© PharmaPress Ltd ISSN 1464-8431

*Peptide-mediated molecular therapeutic delivery systems have recently emerged as an alternative means to effectively substitute or augment present gene therapy technologies, eg, TAT, VP22, engineered peptides. These systems show great promise for the elimination of the main bottleneck to safe, efficient, targeted gene therapy delivery and are able to efficiently introduce DNA, antisense peptide nucleic acids, oligonucleotides, small molecules and proteins into cells both in vitro and in vivo. They are versatile and easily designed to incorporate a number of specific attributes required for efficient cargo delivery. A fundamentally new property of these moieties will allow the therapeutic intervention in the biochemistry of the target cell without the need to alter its genome.*

**Keywords** Antennapedia, membrane translocation, molecular engineering, non-viral delivery, TAT, VP22

### Introduction

Each cell in the body has a specific function and fate. Regulated gene expression determines the character of the cells in each organ, as well as their proper function. Residing within every somatic cell of the body is the complete genetic information required to make an entire organism. There are more than 100,000 genes in the human genome, but only a subset of genes are active in any cell at any given time. The correct regulation in temporal and spatial expression of the DNA blueprint is critical for proper cellular function. Disregulation of the genetic program by mutations in DNA usually results in pathology (eg, cardiovascular, cystic fibrosis, hemophilia and cancer).

The standard paradigm of somatic cell gene therapy is the ability to transfer genetic material into a non-germ diseased cell to induce a permanent cure. Gene therapy is especially

appropriate in well characterized, monogenic diseases, for example, where the specific mutation is known and conventional pharmacological approaches are insufficient, ie, adenosine deaminase deficiency (ADA), hemophilia, cystic fibrosis, Duchenne muscular dystrophy, sickle cell and lung disease [1-5]. Tailoring the treatment regime to specific molecular causes increases the therapeutic index of treatment and reduces its adverse side effects. This is the driving motivation behind gene therapy today.

### The current problems and the ideal system

The lack of predictable safety and efficacy standards in somatic gene therapy systems, has brought the whole field to a crossroads. Replication-incompetent viruses, naked DNA injection and liposomal agents have been the predominant means of genetic transfer. To date, there has been little lasting impact in the typical practice of medicine conferred by these gene therapy technologies. The crux of today's gene therapy dilemma is still the same as it has always been: efficient, safe, targeted delivery and persistent gene expression [1-6].

In light of the safety concerns and efficacy issues encountered with viral transduction [<http://www.med.upenn.edu/ihgt/jesse.html>], [<http://www.med.upenn.edu/ihgt/findings.html>], peptide-based gene delivery agents are emerging as alternatives for safer *in vivo* delivery. The main attraction of these peptide systems is their versatility. Peptide-based delivery systems have the ability to deliver therapeutic proteins, bioactive peptides, small molecules and any size nucleic acids. The use of these agents allows the researcher to intervene at multiple levels in the cells genetics and biochemistry and is a fundamental new technology in the gene therapy field [7-8].

Peptide delivery agents are more like traditional pharmacological drugs than gene therapy vectors. With the past to guide us, a critical re-evaluation of the best characteristics for an ideal delivery system is in order. The desirable features may include the items displayed in Table 1. Peptide-based systems seem to have many of these features inherent in their design, or can be easily engineered to encompass them.

Table 1. Desirable features for a peptide-based delivery system.

Redundant safety features
Efficient transit into cells <i>in vivo</i>
Efficient, stable delivery of undegraded cargo into the nucleus
No size constraints on cargo
Aid in regulated, appropriate and sustained expression of any cargo gene
Amenable to molecular design and engineering
Easily and inexpensively produced at high purity
Efficient targeting
Non-immunogenic, antigenic or inflammatory
Adaptable to any cell type of cell or tissue
Non-toxic and biodegradable
Easy, non-invasive administration
Sufficient serum half-life

### State-of-the-art of gene delivery systems

Viral delivery systems have been state-of-the-art for some time now. The most mature viral technologies are based upon engineered retroviruses, adenoviruses and adeno-associated viruses. Many adenoviral gene therapy protocols rely on the vascular system to deliver virus to the liver by intravenous (iv) injection, or transduction by inhalation. Severe adverse events can and sometimes do occur with direct injection of adenovirus. These adverse events are usually a result of direct immunogenicity and antigenicity of the virus or viral proteins. Almost half of the human population has circulating antibodies to adenovirus. Thus many humans are already 'primed' to resist adenovirus-based therapies. These problems and DNA cargo size constraints have hampered the effective use of these vectors in the clinic.

Liposome delivery systems are another mature technology for gene delivery. Liposomes are synthesized lipid micelle forms, which can incorporate DNA and proteins. Mixed micelles of positively charged and neutral lipids bind electrostatically with the backbone of the DNA to be delivered. This complex shields the cargo from degradation and helps transit across the lipid bilayers of cells. There is inherent competition in the assembly of micelles and the subsequent unpacking of their DNA cargo. This results from the affinity of the lipid head group for the DNA. This lipid interaction can hamper gene transit to the nucleus from the cytosol and therefore expression. Liposome-DNA preparations injected into the tail vein of rodents typically elicit a transient bolus of expression. In many experiments, the lipid is rapidly absorbed onto serum proteins in the liver. Lipid toxicity, lack of targeting and the lack of long-term expression are problems associated with this gene therapy technology. However, new preparations of lipids have addressed some of the toxicity and targeting issues, but long-term *in vivo* expression of cargo alleles remains transient.

Other non-viral delivery systems have been investigated as gene therapy delivery modalities. Delivery of genes and other molecules into cells can also be accomplished by micro-injection, electroporation, encapsulation within polymers, or other physical means. There have been other non-viral delivery alternatives designed and tested in the last few years. These mainly rely on organic polymer technologies. In such systems the polymer backbone is conjugated with biological peptides, such as RGD motifs, which facilitate the delivery [9-12]. It is still too early to assess their feasibility for clinical human gene therapy due to their relatively complex manipulation, lack of specificity, potential cellular toxicity and immunogenicity/antigenicity.

Most gene therapy agents that are in use today have only some of the attributes listed in Table 1. Not every feature on the list needs to be included for a locally effective treatment. For example, a small number of cells transduced by the gene therapy vector can elicit tumor regression. Transient expression of herpes simplex virus thymidine kinase (TK) in part of a tumor allows the killing of the lesion by the prodrug gancyclovir. The TK protein leaks out of dead and dying cells and is then taken up by neighboring tumor cells. This makes the untransduced cells equally susceptible to

death because of gancyclovir's toxicity. The bystander effect can allow killing of the majority of the tumor by transducing a small portion with a prodrug activator gene.

### The early promise and the reality

In the early 1990s retroviral gene therapy protocols were used to treat ADA patients with limited success [13-15]. These trials typically relied on the ability of engineered retroviruses to efficiently enter and establish themselves in dividing cells *ex vivo*. Cells were transplanted back into the patient and the ADA gene product was expressed. The patients transiently expressed enough ADA protein for a time and then the expression decayed to non-therapeutic levels requiring the resumption of standard treatment regimes (the iv infusion of ADA protein). Complications encountered in this trial included the possibility of initiating cancer by insertional mutagenesis of the host cell with retroviruses and the lack of sufficient long-term expression of the transferred allele. Lack of expression of the transferred gene resulted from several problems including: inefficient transduction, regrafting, immune factors and transcriptional silencing of the viral promoters by methylation.

### Peptide-mediated delivery

Many peptide- and protein-mediated delivery systems have been reported (Table 2) [16-20,21,22,23,24,25,26,27,28,29-34]. However, several barriers to their use as delivery agents exist, such as degradation by cellular proteases and nucleic acids by nucleases *in vivo*, efficient uptake into cells in the absence of specific receptors and transporters and escape from the endosome/lysosomal compartment. These are surmountable obstacles; which are amenable to rapid, rational engineering design and analysis.

The transit of peptides and proteins across lipid bilayers is generally energetically unfavorable. However, there are several naturally occurring proteins, which can pass across the membrane unencumbered by the need for specific transporter schemes. Some examples of this type of protein are antennapedia (*Drosophila*), Tat (HIV) and VP22 (herpes). Several peptides derived from these (and other) protein sequences are available that can efficiently enter mammalian cells, transit to the nucleus or carry molecular cargoes into the cell (Table 2).

### HIV Tat protein

The 86 amino acid HIV transcriptional activator protein Tat, can be synthesized in one cell and then released and transited into a neighboring cell [35,36]. A receptor-mediated event is not required for TAT to pass into a neighboring cell. HIV-1, as well as all other lentiviruses, encode a potent Tat. This protein binds to specific regions of the virus genome (tar) and recruits cellular factors to increase the efficient transcription of the proviral genome. Tat is an important virulence factor of HIV infection. The Tat protein has three domains: the cysteine-rich, basic and integrin-binding regions. This protein has many effects on various host cells including being pro-angiogenic in endothelial cells [37,38].

Table 2. Peptides used in DNA, oligonucleotides, peptide and protein delivery.

Name	Sequence (N→C)	Size (kDa)	Reference
Tat	YGRKKRRQRRR	11	[21••]
ANTp	RQIKIWQNRRMKWKK	16	[22••]
W/R	RRWRRWRRWRRWRR	16	[23]
NLS*	TPPKKKRKVEDP	12	[16]
AlkCWK <sub>11</sub>	OWKKKKKKKKKKKKKKKK	20	[20]
DiCWK <sub>11</sub>	K <sub>11</sub> WCCWK <sub>11</sub>	40	[20]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27	[24]
Dipalytic	GLFEALEELWEAK	13	[25••]
K <sub>16</sub> RGD	K <sub>16</sub> GGCRGDMFGCAK16RGD	46	[26]
P1	K <sub>16</sub> GGCMFGCGG	25	[27]
P2	K <sub>16</sub> OCRRARGDNPPDRCT	31	[27]
P3	KKWKMRNRNQFWVKVGRBAK (B) bA	20	[29••]
P3a	VAYISRGGVSTYSDTVKGRFTROKYNKRA	30	[29]
P9.9	IGRIDPANGSKTKYAPKFQDKATRSNYYGNSSPS	32	[29]
P1ae	PLAEIDGIELTY	12	[30]
Kplae	K <sub>16</sub> GGPLAEIDGIELGA	30	[30]
ckplae	K <sub>16</sub> GGPLAEIDGIELCA	30	[30]
MGP	GALFLGFLGGAAGSTMGAWSQPKSKRKV	27	[31,32]
HA2	WEAK (LAKA) LAKH (LAKA) LKAC	28	[33]
LARL <sub>4</sub>	(LARL) <sub>4</sub> -NH <sub>2</sub>	24	[33]
Hel-11-7	KLLKLLKLLKLLKLL	16	[33]
KK	(KKKK) <sub>1</sub> GGC	NA	[17]
KWK	(KWKK) <sub>1</sub> GCC	NA	[17]
RWR	(RWR) <sub>1</sub> GCC	NA	[17]
Lollipop	K5K2 (K4) (K6) GGGKKKK-NLS	NA	[34]

Single letter code for amino acids are used for all peptides. The sizes range from 11 to 46 kDa. There are no obvious consensus sequences or conserved motifs. Some have positively charged residues for DNA binding, and clusters of hydrophobic residues, especially those with aromatic side chains, which presumably further condense DNA and facilitate membrane translocation. This list is by no means complete.

\*NLS = nuclear localization signal; only one NLS is listed here although there are many variations of NLS that have been described. Derossi *et al* [22••] described a table which listed many variations of ANTp and its derivatives. Plank *et al* [17] have listed many more branched peptides; only three examples are listed here.

NA = Not applicable

A Tat-derived peptide that can traffic across the membrane is the protein transduction domain (PTD); which is only 11 amino acids long. Protein translational fusions with this sequence (YGRKKRRQRRR) transit efficiently across the mammalian cell membrane *in vitro* and *in vivo* into the nucleus. 50 Proteins from 15 to 120 kDa have been tested and all enter target human and murine cells efficiently [21••,39]. Additionally, bioactive peptides with the PTD appended remain biologically active with the appended transit sequence. These chemically synthesized peptides can rapidly pass into cells, accumulating in the nucleus.

Translational fusions of the 120 kDa  $\beta$ -galactosidase with Tat have been synthesized and injected into the peritoneum of mice [21••]. The remarkable results of these studies indicates that the Tat-tagged protein is rapidly taken up by all the cells and tissues in the mouse, including the brain. The blood-brain barrier is usually only accessible to small, lipophilic molecules.  $\beta$ -Galactosidase crosslinked to 35 amino acid Tat peptide can also be efficiently internalized. In both of these studies, the cell-associated  $\beta$ -galactosidase protein was active in *in situ* and FACS analysis. Interestingly, the crosslinked  $\beta$ -galactosidase was reported not to accumulate in the brain of mice.

The discrepancy between the earlier report by Fawell *et al* [40] and the recent report by Schwarze *et al* may be a result of differing sample preparation times or different means of

preparing the  $\beta$ -galactosidase protein. The sampling time reported by Fawell *et al* [40] was about 20 min, while a recent report quoted several hours [21••]. Schwarze *et al* describe a method for  $\beta$ -galactosidase preparation which may yield 'misfolded' protein [21••], but the 'molten globule' appearance of the  $\beta$ -galactosidase may aid in its transit. Schwarze *et al* used intraperitoneal delivery rather than tail vein injections [21••]. Further investigations into the mode of entry and biophysics of Tat chimeras in transit through biological membranes are warranted.

Unfortunately, there is no apparent specificity in the transfer of the  $\beta$ -galactosidase protein into any cell type. All cells seem to incorporate the  $\beta$ -galactosidase fusion [21••]. If this method were to be used to deliver the gancyclovir prodrug activator, herpes thymidine kinase protein into cells, then unwanted collateral cell death would result. The inclusion of a dominant targeting domain in the chimera could eliminate this type of damage.

### *Drosophila* homeoprotein antennapedia

The *Drosophila* homeotic protein antennapedia (ANTp) is capable of transiting across the membranes of animals and acting at a distance [22••]. The biology of ANT has been reviewed by Dorn *et al* [41••]. Briefly, homeobox genes specify spatial units of body plan. There is a 180 bp homeobox sequence upstream of the genes which encode a

DNA binding site for the homeotic gene products. Homeotic genes are helix turn helix proteins. A derivative of 60 amino acids of ANTP can translocate across cell membranes and bind to the homeobox sequence. The 16 amino acids (RQIKIWFQNRRMKWKK) of the third helix of ANTP (part of the 60 amino acids) have been identified as the minimal unit which can cross the membrane bilayer and eventually accumulate in the nucleus. Interestingly, the presence of a neural cell adhesion molecule (NCAM) linked to  $\alpha$ -2,8-polysialic acid increases the efficiency of transit 4-fold. The internalization of ANTP is energy-independent and functions efficiently at 4 °C, suggesting that the translocation mechanism may be based on amphiphilicity. Different derivatives of this peptide have been synthesized and tested for entry and are reviewed by Derossi *et al* [22]. The results suggest that the entry of ANTP relies on key tryptophan, phenylalanine and glutamine residues. Furthermore, the retroinverse and all D-amino acid forms also are translocated efficiently. Therefore, a specific helical structure is not a prerequisite for membrane translocation entry. A proposed means of ANTP entry is that there may be a two-step interaction. The first step is the electrostatic approximation of the positively charged residues to the negatively charged cell surface. Secondly, the hydrophobic residues may facilitate interaction and translocation with the membrane. The presence of  $\alpha$ -2,8-polysialic acid increases the net charge of the cell surface, aiding this type of interaction. The association of the positively charged ANTP with the negatively charged sialic acid chains would yield a neutral sialic acid and result in a local pH change at the cell surface. This pH change might induce the insertion of the ANTP into the hydrophobic membrane, which would concentrate the ANTP on the cell surface and allow hydrophobic forces to take hold, permitting entry. Exit of the ANTP-sialic acid complex would occur as a result of transition to the more neutral pH of the cytosol. This buffering pH shift would result in the release of ANTP. Entry is not dependent on sialic acid and ANTP transduces many cell types that do not express this material. The passage of ANTP from the cytosol to the nucleus can occur by passive diffusion. However, the rate of nuclear entry and the abundance of basic residues infer the presence of a nuclear localization sequence (NLS). The successful transduction of DNA that is larger than oligonucleotides has not yet been reported, but many bioactive peptides have been shown to traffic into cells when the ANTP sequence is added to it (Table 2).

### Herpes virus VP22

Herpes virus VP22 tegument is another unusual protein molecule, which can efficiently traffic across cell membranes [42,43,44,45-47]. It is so efficient that expression in a small population of cells allows VP22 protein to traffic intercellularly to all cells of the culture. This protein concentrates in the nucleus and binds to chromatin. VP22 appears to traffic through the membrane via non-classical endocytosis and can enter cells regardless of GAP junctions and physical contacts [45]. Transit of VP22 is susceptible to actin cytoskeleton disruption. This protein colocalizes to cellular microtubules and causes them to reorganize into bundles. VP22 is also the target of phosphorylation. During viral infection, the VP22 that accumulates in the nucleus is phosphorylated, while that found in the cytoplasm is not.

This phosphorylation is likely a result of casein kinase II activity, as VP22 has four amino proximal serine residues in consensus sites. The last 34 amino acids are critical for transit. It is important to note that only carboxyl-terminal translational fusion proteins can be carried into cells. The biological activities of several proteins (p53, GFP, thymidine kinase and others) have been investigated as translational fusions with VP22 [44,45,46]. The VP22 cloning and expression system is now commercially available to investigators (Invitrogen Inc, <http://www.invitrogen.com>).

Approximately half of all cancers have diminished p53 activity [48,49]. Supplementing a 'good' copy of the gene product to these cells could induce them to undergo apoptosis and kill the tumor. The VP22-p53 chimerical protein retained its ability to spread to between cells and its proapoptotic activity, and had a widespread cytotoxic effect in p53 negative human osteosarcoma cells [44]. Normal and supernormal levels of p53 are not usually harmful to normal cells. Unwanted side effects should be minimal with this type of cancer treatment.

HSV-TK linked in frame with VP22, was transported between the cells of a neuroblastoma culture and had TK activity. These cells are GAP junction negative [46]. Treatment of tissue culture cells transfected with TK protein alone, or VP22-TK protein with gancyclovir demonstrated that only the VP22-TK-treated cells died. Tumors established with a neuroblastoma cell line expressing VP22-TK regressed, while those expressing TK alone did not [46]. These results suggest that this novel method of cancer therapy holds promise in the clinic.

VP22-GFP (DNA expression plasmid) was transfected into COS7 cells. These cells were then cocultured with target C2C12 myotubes. The C2C12 cells were permissive to efficient VP22-GFP entry [46]. Terminally differentiated skeletal muscle cells were also transduced with a VP22-GFP protein, which suggests that mitosis is not a requirement for efficient entry [46].

Unfortunately, immunogenicity may be a problem with VP22 chimeras. VP22-reactive T-cells may have a role to play in the control of recurrent HSV infection. The antiviral functions of infiltrating CD4-bearing T-cells may include cytotoxicity, inhibition of viral growth, lymphokine secretion and support of humoral and CD8 responses. Viral VP22 and dUTPase are known to be T-cell antigens [50]. Analysis of the clonal reactive attributes of T-cells recovered from herpetic lesions from several patients proved this point. Some VP22 CD4 T-cells exhibited cytotoxic activity against HSV infected cells. VP22 and dUTPase are now being evaluated as possible candidates for protective vaccination epitopes. While many of the attributes of VP22 are laudable, the fact that there might be a vigorous immune reaction to the chimera *in vivo* should be considered carefully when using this method in human gene therapy.

### Other peptide-mediated gene delivery systems

Peptide-mediated delivery systems including transportan, MPG, SCWKn, (LARI)n, HA2, RGD, NLS, oligomer and others (Table 2) have been reported. Transportan, is a chimerical peptide derived from the first 12 residues of

galanin connected by an  $\epsilon$ -amino of a lysine with a 14 residue mastoparan. Mastoparan can effectively deliver oligonucleotides into cells in culture and animals to block cell signaling activities [24]. The MPG peptide is also a chimera; it is comprised of part of the HIV gp41 protein and the NLS of SV40 large T-antigen. MPG can act as a potent gene delivery agent [31,32]. The cell adhesion motif RGD linked with oligolysine has been reported to deliver genes into cells [26]. Alterations of the region bounding the RGD motif also allow cell type-specific targeting.

## Conclusions

Peptide- and protein-mediated gene delivery systems are currently not the state-of-the-art choice for delivery. However, these systems are extremely versatile and amenable to rational design and modification. Peptides can also include many unique features like cell tropism and flexible cargo delivery. Problems of antigenicity inherent in peptide system design can be eliminated or minimized. ANTP has been shown to be only weakly immunogenic in mice. *In vitro* protein/peptide evolution and *in vitro* phage-display technologies [51,52,53, <http://www.phyles.com>] allows the discovery of additional peptides/proteins that are more efficient, safe and specific for targeted delivery. We expect that out of this emerging field, peptide systems will play an increasing role in targeted molecular therapeutics and gene therapy.

## Acknowledgement

This work is supported in part by grants from NIHHR21, NSF to the Biotechnology Process Engineering Center at MIT and the US Army Research Office.

## References

- of outstanding interest
- of special interest

1. Anderson WF: Human gene therapy. *Nature* (1998) 392(Suppl 6679):25-30.  
•• This is a thoughtful review of gene therapy, which outlines the prospects and problems of gene therapy delivery.
2. Ennist DL: Gene therapy for lung disease. *Trends Pharmacol Sci* (1999) 20:260-266.
3. Vassalli G, Dichek DA: Gene therapy for arterial thrombosis. *Cardiovasc Res* (1997) 35:459-469.
4. Lewis BS, Flugelman MY, Weisz A, Keren-Tal I, Schaper W: Angiogenesis by gene therapy: A new horizon for myocardial revascularization? *Cardiovasc Res* (1997) 35:480-497.
5. Yla-Herttuala S: Vascular gene transfer. *Curr Opin Lipidol* (1997) 8:72-76.
6. Gorecki DC, MacDermot KD: Gene therapy: Panacea or placebo? II. Main applications of gene therapy. *Arch Immunol Ther Exp* (1997) 45:375-381.
7. Norman TC, Smith DL, Sorger PK, Drees BL, O'Rourke SM, Hughes TR, Roberts CJ, Friend SH, Fields S, Murray AW: Genetic selection of peptide inhibitors of biological pathways. *Science* (1998) 285:591-595.  
• This paper describes a method for selecting biologically interesting peptide inhibitors of cellular processes.

8. Aramburo J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A: Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* (1999) 285:2129-2133.  
• Demonstrates the efficacy and utility of peptide intervention in medically relevant cell biology.
9. Erbacher P, Remy JS, Behr JP: Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther* (1999) 6:138-145.
10. Ferrari S, Pettenazzo A, Garbati N, Zecchello F, Behr JP, Scarpa M: Polyethylenimine shows properties of interest for cystic fibrosis gene therapy. *Biochim Biophys Acta* (1999) 1447:219-225.
11. Romanczuk H, Galer CE, Zabner J, Barsomian G, Wadsworth SC, O'Riordan CR: Modification of an adenoviral vector with biologically selected peptides: A novel strategy for gene delivery to cells of choice. *Hum Gene Ther* (1999) 10:2615-2626.
12. Pack DW, Putnam D, Langer R: Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnol Bioeng* (2000) 67:217-223.
13. Hershfield MS: Adenosine deaminase deficiency: Clinical expression, molecular basis and therapy. *Semin Hematol* (1998) 35:291-296.
14. Hoogerbrugge PM, von Bausechem VW, Kaptein LC, Einemund MP, Valerio D: Gene therapy for adenosine deaminase deficiency. *Br Med Bull* (1995) 51:72-81.
15. Hoogerbrugge PM, van Bausechem VW, Fischer A, Debré M, le Deist F, Perignon JL, Morgan G, Gaspar B, Fairbanks LD, Skeoch CH, Mosseley A, Harvey M, Levinsky RJ, Valerio D: Bone marrow gene transfer in three patients with adenosine deaminase deficiency. *Gene Ther* (1996) 3:179-183.
16. Zanta MA, Belguise-Valladier P, Behr JP: Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci USA* (1995) 92:91-95.
17. Plank C, Tang MX, Wolfe AR, Szoka FC: Branched cationic peptides for gene delivery: Role of type and number of cationic residues in formation and *in vitro* activity of DNA polyplexes. *Hum Gene Ther* (1999) 10:319-332.
18. Knight A, Carvajal J, Schneider H, Coutelle C, Chamberlain S, Fairweather N: Non-viral neuronal gene delivery mediated by the HC fragment of tetanus toxin. *Eur J Biochem* (1999) 259:762-769.
19. Ziady AG, Ferkoi T, Gerken T, Dawson DV, Perlmutter DH, Davis PB: Ligand substitution of receptor targeted DNA complexes affects gene transfer into hepatoma cells. *Gene Ther* (1998) 5:1685-1697.
20. Wadhwa MS, Collard WT, Adami RC, McKenzie DL, Rice KG: Peptide-mediated gene delivery: Influence of peptide structure on gene expression. *Bioconjug Chem* (1997) 8:81-86.
21. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF: *In vivo* protein transduction: Delivery of a biologically active protein into the mouse. *Science* (1999) 285:1569-1572.  
•• This paper demonstrates the power and utility of the Tat fusion protein system to deliver  $\beta$ -galactosidase in the mouse.
22. Derossi D, Chassaing G, Prochiantz A: Trojan peptides: The penetratin system for intracellular delivery. *Trends Cell Biol* (1998) 8:84-87.  
•• A complete review of antennapedia-mediated delivery.



23. Williams EJ, Dunican DJ, Green PJ, Howell FV, Derossi D, Walsh FS, Doherty P: Selective inhibition of growth factor-stimulated mitogenesis by a cell-permeable Grb2-binding peptide. *J Biol Chem* (1997) 272:22349-22354.
24. Pooga M, Soomets U, Halilbrink M, Valkna A, Saar K, Rezaei K, Kahi U, Hao JK, Xu XJ, Wiesentfeld-Hallin Z, Holdelt T, Banfal T, Langel U: Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission *in vivo*. *Nature Biotechnol* (1998) 16:857-861.
25. Mahato RI, Smith LC, Rolland A: Pharmaceutical perspectives of non-viral gene therapy. *Adv Genet* (1998) 41:95-155.
- This review describes the state-of-the-art in non-viral gene delivery. It also mentions peptide-mediated modes of transfection.
26. Harbottle RP, Cooper RG, Hart SL, Ladhoff A, McKay T, Knight AM, Wagner E, Miller AD, Coutelle C: An RGD-oligolysine peptide: A prototype construct for integrin-mediated gene delivery. *Hum Gene Ther* (1998) 9:1037-1047.
27. Shawing L, Collins L, Lightman SL, Hart S, Gustafsson K, Fabre JW: A non-viral vector system for efficient gene transfer to corneal endothelial cells via membrane integrins. *Transplantation* (1997) 64:763-769.
28. Aldrian-Herrada G, Desarmenien MG, Oroel H, Boissin-Agasse L, Mery J, Brugidou J, Rabie A: A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. *Nucl Acid Res* (1998) 26:4910-4916.
- This article demonstrates that the helical nature of the ANTP peptide is not required for entry. Additionally, the delivery and the utility of antisense PNAs into cells is greatly increased by conjugation with ANTP.
29. Avrameas A, Ternynck T, Gasmi L, Buttin G: Efficient gene delivery by a peptide derived from a monoclonal anti-DNA antibody. *Bioconjug Chem* (1999) 10:87-93.
30. Schneider H, Harbottle RP, Yokosaki Y, Jost P, Coutelle C: Targeted gene delivery into  $\alpha 9 \beta 1$ -integrin-displaying cells by a synthetic peptide. *FEBS Lett* (1999) 458:329-332.
31. Morris MC, Chaloin L, Mery J, Heitz F, Divita G: A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucl Acid Res* (1999) 27:3510-3517.
32. Morris MC, Vidal P, Chaloin L, Heitz F, Divita G: A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucl Acid Res* (1997) 25:2730-2736.
33. Niidome T, Takaji K, Urakawa M, Ohmori N, Wada A, Hirayama T, Aoyagi H: Chain length of cationic  $\alpha$ -helical peptide sufficient for gene delivery into cells. *Bioconjug Chem* (1999) 10:773-780.
34. Singh D, Bisland SK, Kawamura K, Gariepy J: Peptide-based intracellular shuttle able to facilitate gene transfer in mammalian cells. *Bioconjug Chem* (1999) 10:745-754.
35. Vives E, Brodin P, Lebloucq B: A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* (1997) 272:16010-16017.
36. Kam J: Tackling Tat. *J Mol Biol* (1999) 293:235-254.
37. Albini A, Barilari G, Benelli R, Gallo RC, Ensoli B: Angiogenic properties of human immunodeficiency virus type 1 Tat protein. *Proc Natl Acad Sci USA* (1995) 92:4838-4842.
38. Tarabotti G, Benelli R, Borzotti P, Rusnati M, Presta M, Giavazzi R, Ruco L, Albini A: Thrombospondin-1 inhibits Kaposi's sarcoma (KS) cell and HIV-1 Tat-induced angiogenesis and is poorly expressed in KS lesions. *J Pathol* (1999) 188:76-81.
39. Ethymiadis A, Briggs LJ, Jans DA: The HIV-1 Tat nuclear localization sequence confers novel nuclear import properties. *J Biol Chem* (1998) 273:1623-1628.
40. Fawell S, Seery J, Dalkh Y, Moore C, Chen LL, Pepinsky B, Barsom J: Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci USA* (1994) 91:664-668.
41. Dom A, Affolter M, Gehring WJ, Leupin W: Homeodomain proteins in development and therapy. *Pharmacol Ther* (1994) 51:155-184.
- A complete review of antennapedia.
42. Elliott G, O'Hare P: Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* (1997) 88:223-233.
- This paper catalogs the functions and localization of VP22 and discusses its possible uses.
43. Elliott G, O'Hare P: Intercellular trafficking of VP22-GFP fusion proteins. *Gene Ther* (1998) 5:149-151.
44. Phean A, Elliott G, O'Hare P: Intercellular delivery of functional p53 by the herpesvirus protein VP22. *Nature Biotechnol* (1998) 16:440-443.
- This paper demonstrates the utility of the VP22 system for novel, molecular therapeutics.
45. Dilber MS, Phean A, Aints A, Mohamed AJ, Elliott G, Edvard Smith CI, O'Hare P: Intercellular delivery of thymidine kinase prodrug activating enzyme by the herpes simplex virus protein, VP22. *Gene Ther* (1999) 6:12-21.
46. Derer W, Easwaran HP, Knopf CW, Leonhardt H, Cardoso MC: Direct protein transfer to terminally differentiated muscle cells. *J Mol Med* (1999) 77:609-618.
47. Luft FC: Can VP22 resurrect gene therapy? *J Mol Med* (1999) 77:575-576.
48. Levine AJ: p53, the cellular gatekeeper for growth and division. *Cell* (1997) 88:323-331.
49. Levine AJ: The tumor suppressor genes. *Annu Rev Biochem* (1993) 62:623-651.
50. Koelle DM, Frank JM, Johnson ML, Kwok WW: Recognition of herpes simplex virus type 2 tegument proteins by CD4 T-cells infiltrating human genital herpes lesions. *J Virol* (1998) 72:7476-7483.
51. Pasqualini R, Ruoslahti E: Organ targeting *in vivo* using phage display peptide libraries. *Nature* (1996) 380:364-366.
- This paper describes a method for selecting peptide sequences, which target different organs. The phage sequences described are useful for targeting specific organs.
52. Roberts RW, Szostak JW: RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc Natl Acad Sci USA* (1997) 94:12297-12302.
53. Roberts RW: Totipotency *in vitro* protein selection using mRNA-protein fusions and ribosome display. *Curr Opin Chem Biol* (1999) 3:268-273.